A long read optimized *de novo* transcriptome pipeline reveals novel ocular developmentally regulated gene isoforms and disease targets

Vinay S. Swamy1, Temesgen D. Fufa2, Ameera Mungale2, Robert B. Hufnagel2, and David M. McGaughey1,✉

1 Bioinformatics Group, Ophthalmic Genetics & Visual Function Branch, National Eye Institute, National Institutes of Health  
2 Medical Genetics and Ophthalmic Genomics Unit, National Eye Institute, National Institutes of Health

✉ Correspondence: [David M. McGaughey <[mcgaugheyd@mail.nih.gov](mailto:mcgaugheyd@mail.nih.gov)>](mailto:mcgaugheyd@mail.nih.gov)

### Short Title (70 characters)

**Computational identification of novel gene isoforms in ocular tissues**

# Abstract

*De novo* transcriptome construction from short-read RNA-seq is a common method for reconstructing mRNA transcripts within a given sample. However, the precision of this process is unclear as it is difficult to obtain a ground-truth measure of transcript expression. With advances in third generation sequencing, full length transcripts of whole transcriptomes can be accurately sequenced to generate a ground-truth transcriptome. We generated long-read PacBio and short-read Illumina RNA-seq data from a human induced pluripotent stem cell-derived retinal pigmented epithelium (iPSC-RPE) cell line. We use the long-read data to identify simple metrics for assessing *de novo* transcriptome construction and optimizing a short-read based *de novo* transcriptome construction pipeline. We apply this pipeline to construct transcriptomes for 340 publicly available short-read RNA-seq samples originating from healthy adult and fetal human retina, cornea, and RPE and identify hundreds of novel gene isoforms and examine their significance in the context of ocular development and disease.

# Author Summary

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             Gene isoforms are the final product of DNA transcription, and are responsible for increasing the transcriptome diversity of biological systems. While over 80,000 isoforms have been identified in humans, many remain undiscovered. Previous work in the field has shown gene isoforms play key roles in a myriad of biological processes, such as development, and furthermore can act as drivers in human disease. We improve upon existing computational methods for identifying novel gene isoforms to create a novel annotation of gene isoforms within the human eye. We show that these novel gene isoforms play a role during the development of the eye and in multiple inherited eye diseases.

# Introduction

             The transcriptome is defined as the set of unique RNA transcripts expressed in a biological system. A single gene can have multiple distinct transcripts, or isoforms, and there are multiple biological processes that drive the formation of these isoforms including alternative promoter usage, alternative splicing, and alternative polyadenylation. Gene isoforms can have distinct and critical functions in biological processes like development, cell differentiation, and cell migration [1], [2], [3]. Alternative usage of isoforms has also been implicated in multiple diseases including cancer, cardiovascular disease, Alzheimer’s disease and diabetic retinopathy [4], [5], [6], [7].

             Accurate annotation of gene isoforms is fundamental for understanding their biological impact. The Gencode human comprehensive transcript annotation (release 28) contains 82335 protein coding and 121500 noncoding transcripts across 19901 genes and 38480 pseudogenes, but previous studies have shown this annotation is incomplete [8], [9]. Some of the first high throughput methods to find novel gene isoforms used short-read (~100bp) RNA-sequencing (RNA-seq) to identify novel exon-exon junctions and novel exon boundaries based solely on RNA-seq coverage [10]. More recently, several groups have developed specialized methods for using RNA-seq to reconstruct the whole transcriptome of a biological sample, dubbed *de novo* transcriptome construction [11],[2], [12].

*De novo* transcriptome construction uses short-read RNA-seq to reconstruct full-length mRNA transcripts. However, a large number of samples are necessary to overcome the noise and short-read lengths of this type of data. Partially because of steadily decreasing sequencing cost, datasets of the necessary size are now available. For example, one of the most comprehensive *de novo* transcriptome projects to date is CHESS, which uses the GTEx data set to construct *de novo* transcriptomes in over 9000 RNA-seq samples from 44 distinct body locations to create a comprehensive annotation of mRNA transcripts across the human body [13], [14]. However, since the GTEx dataset does not include samples from any ocular tissues, the CHESS database remains an incomplete annotation of the human transcriptome.

             Despite the increasing number of *de novo* construction methods developed, there is no gold standard to evaluate the precision and sensitivity of *de novo* transcriptome construction on real (not simulated) biological data. Long-read sequencing technologies provide a potential solution to this problem as long-read sequencing can capture full length transcripts and thus, can be used to identify a more comprehensive range of gene isoforms. While previous iterations of long-read sequencing technologies typically had higher error rates, the new PacBio Sequel II system sequences long-reads as accurately as short-read based sequencing [15].

             We propose that long-read based transcriptomes can serve as a ground truth for evaluating short-read based transcriptomes. In this study, we used PacBio long-read RNA sequencing to inform the construction of short-read transcriptomes. We generated PacBio long-read RNA-seq along with matched Illumina short-read RNA-seq data in human retinal pigmented epithelium (RPE) cells derived from induced pluripotent stem cells (iPSC) using in vitro differentiation. We then designed a rigorous StringTie-based pipeline to maximize the concordance between short and long-read *de novo* transcriptomes (Figure 1).

             Finally, we applied this optimized pipeline to a data set containing 340 human ocular tissue samples compiled from mining previously published short-read RNA-seq data [16]. We built transcriptomes for three major ocular tissues: cornea, retina, and RPE, using RNA-seq data from both adult and fetal tissues to create a high-quality pan-eye transcriptome. In addition to ocular samples, we used 877 samples from the GTEx dataset to construct transcriptomes for tissues in 44 other locations across the body(Figure 1). We refer to each distinct body location as a subtissue here after.

             We used our gold-standard informed pan-eye *de novo* transcriptome to reveal hundreds of novel gene isoforms in the eye and analyze their potential impact on ocular biology and disease. We provide transcript annotation derived from our *de novo* transcriptomes as a resource to other researchers through an R package.

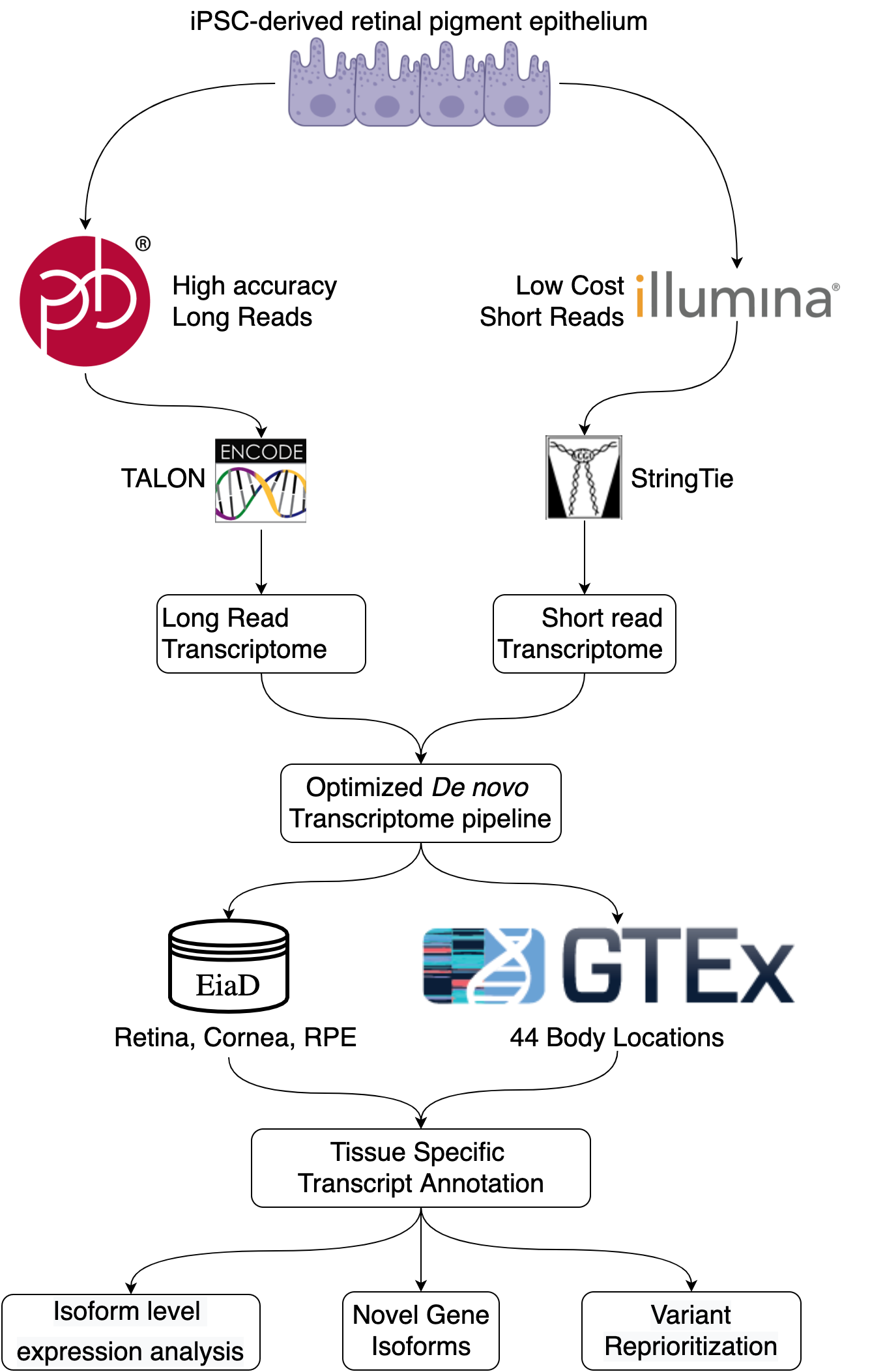


Figure 1. Workflow for long-read informed *de novo* transcriptome construction and analysis

# Results

## Long-read PacBio RNA sequencing guides short-read *de novo* transcriptome construction

             To evaluate the accuracy of short-read transcriptome construction, we first generated PacBio long-read RNA-seq data (n=1) and Illumina short-read RNA-seq data (n=14) from iPSC-RPE. These cells were differentiated using an optimized protocol, and thus minimal biological variation is expected [17], [18]. We used these sequencing data to construct a long-read transcriptome and a short-read transcriptome. We found 1163239 and 366888 distinct transcripts in our long-read and short-read transcriptomes, respectively.

             In our initial comparison between short and long-read transcriptomes, we noticed a low transcriptome construction accuracy (see Methods) of 0.208. When we examined the transcript lengths of each build we saw that the two methods show very different transcript length distributions for both novel and previously annotated transcripts, with the short-read build was comprised mostly of smaller transcripts (Fig 2A). As the PacBio data was generated using two different libraries for 2000 bp and >3000 bp transcripts, we expected an enrichment for longer transcripts in the PacBio data set (Supplemental Figure 1). To assess accuracy relative to transcript length, we grouped transcripts by length in 1000 bp intervals, and compared accuracy between each group. We found that accuracy significantly improves for transcripts longer than 2000 bp. The construction accuracy is 0.426 and 0.137 for transcripts above and below 2000 bp, respectively.

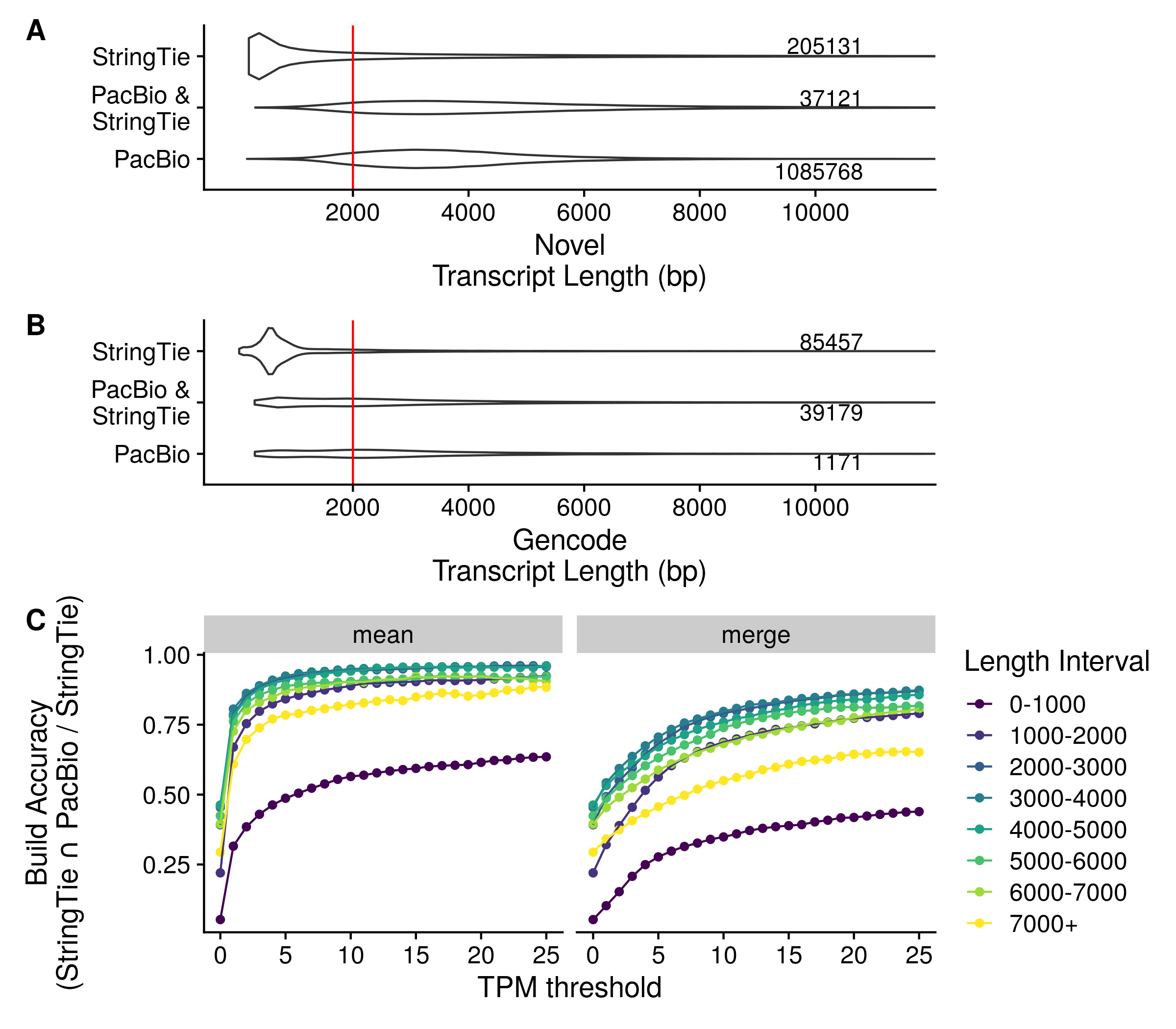


Figure 2. Transcript length and expression dictate transcriptome construction accuracy. A,B) Distributions of novel(A) and previously annotated(B) transcript lengths between PacBio (long-read) and Stringtie (short-read) transcriptomes. Each distribution is labeled with the total number of transcripts in the distribution C) Short-read construction accuracy stratified by transcript length at different Transcripts Per Million (TPM)-based transcript exclusion thresholds. The “merge” method follows the protocol for constructing transcriptomes outlined by the StringTie authors and keeps any transcripts expressed above a specific TPM threshold in at least one sample. The “mean” method used by our pipeline keeps transcripts whose average expression across all samples is above a specific TPM threshold.

             We experimented with various methods to remove spurious transcripts and improve construction accuracy. We first removed transcripts that were expressed <1 TPM in at least one sample as outlined in StringTie’s recommended protocol [19]. This improved construction accuracy to 0.475 for transcripts longer than 2000bp and 0.212 for transcripts shorter than 2000bp. As this accuracy was still fairly low, we tried different filtering schemes but found that the simplest approach with high performance was to retain transcripts that had an average TPM above a specific threshold (Fig 2C). In our downstream pipeline we keep transcripts that have at least an average of 1 TPM across all samples from the same subtissue because this threshold achieved a build accuracy of 0.772 for transcripts longer than 2000 bp and retained 48470 transcripts within our short-read RPE dataset.

## Thousands of novel gene isoforms are detected in human subtissue-specific transcriptomes

             We built transcriptomes from 340 publicly available ocular tissue RNA-seq samples curated in EiaD using an efficient Snakemake pipeline [20]. We included non-disease, non-perturbed adult and fetal samples from cornea, retina, and RPE tissues, mined from 29 different studies (Table 1). Our fetal tissues consist of both real human fetal tissues and human iPSC-derived tissue, as stem cell-derived tissue has been shown to closely resemble fetal tissue [21], [22]. We include our 14 iPSC-RPE samples originally used to develop our pipeline within this larger set of fetal RPE samples. To more accurately determine the tissue specificity of novel ocular transcripts, we supplemented our ocular data set with 877 samples from 44 subtissues across 22 major tissues from the GTEx project and constructed transcriptomes for each of these subtissues [13].

| Tissue | Source | Samples | Studies | Transcriptome Count |
| --- | --- | --- | --- | --- |
| RPE | Adult | 48 | 4 | 32,012 |
| RPE | Fetal | 49 | 7 | 49,967 |
| Retina | Adult | 105 | 8 | 49,714 |
| Retina | Fetal | 89 | 6 | 66,255 |
| Cornea | Adult | 43 | 6 | 51,469 |
| Cornea | Fetal | 6 | 2 | 59,408 |

Table 1. Ocular sample dataset overview and transcriptome count. Transcriptome count is defined as the number of unique transcripts expressed in a given tissue type

             After initial construction of transcriptomes, we found 183442 previously annotated transcripts and 6241675 novel transcripts detected in at least one of our 1217 samples. We define a novel transcript as any transcript whose set of exons and introns do not exactly match that of an annotated transcript within the Gencode, Ensembl, UCSC, and Refseq annotation databases [8], [23], [24]. After using the filtering methods described above, we merged all subtissue specific transcriptomes into a single final transcriptome which contains 252983 distinct transcripts with 87592 previously annotated and 165391 novel transcripts, and includes 114.9 megabases of previously unannotated genomic sequence (Table 1). We refer to the final pan-body transcriptome as the DNTX annotation hereafter.

             We split novel transcripts into two categories: novel isoforms, which are novel variations of known genes, and novel loci, which are previously unreported, entirely novel regions of transcribed sequence (Fig 3B). Novel isoforms are further classified by the novelty of their encoded protein: isoforms with novel open reading frame (ORF), novel isoforms with a known ORF, and isoforms with no ORF as noncoding isoforms (Fig 3A). The number of distinct ORFs was significantly less than the number of transcripts, with 43279 previously annotated ORFs and 46226 novel ORFs across all subtissues. Furthermore, across all subtissues there was an average of 10393 novel isoforms and 3716 novel ORFs.

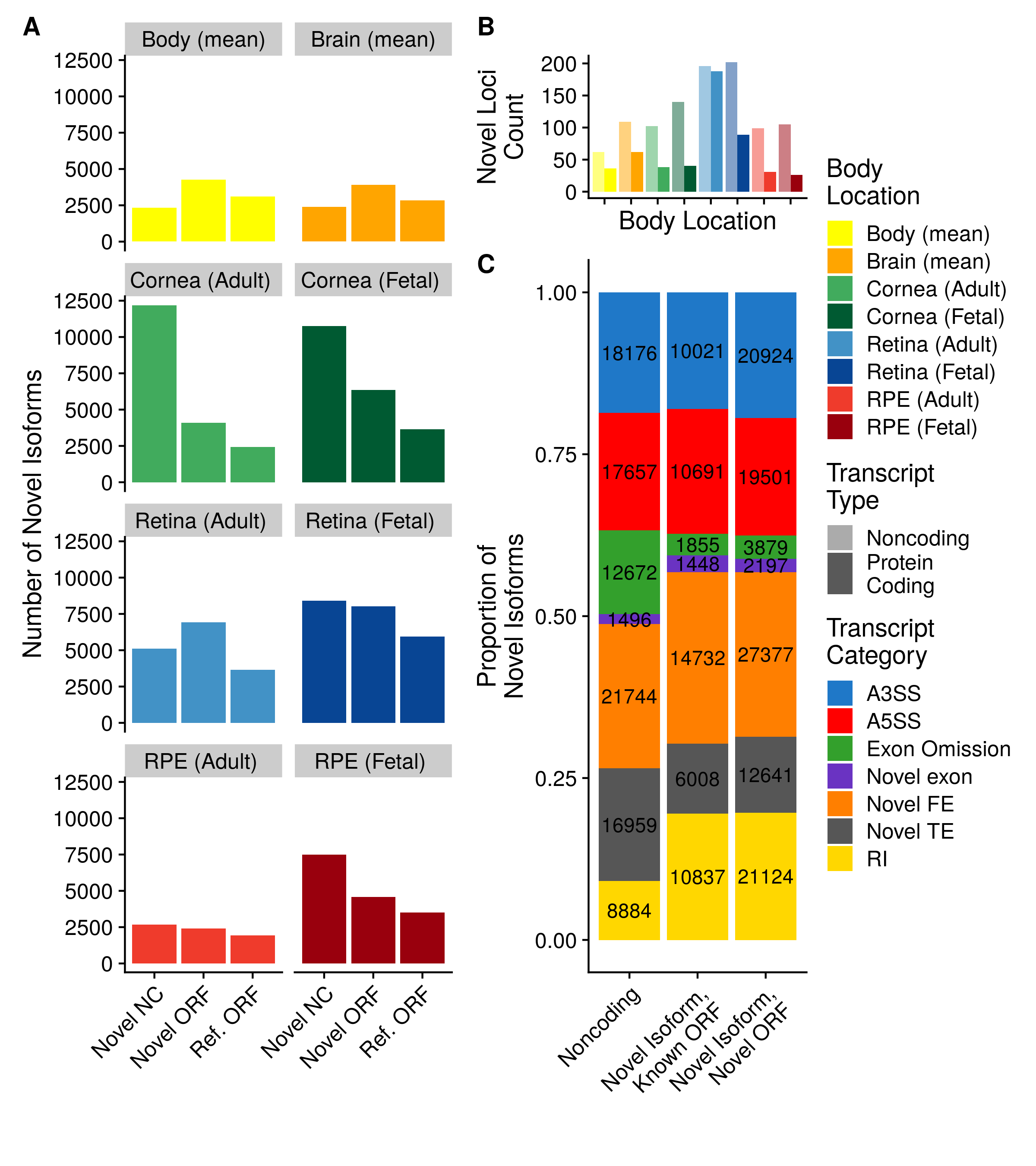


Figure 3. Overview of novel isoforms. A) Number of novel gene isoforms, grouped by transcript type. Brain and body represent an average of 13 and 34 distinct subtissues, respectively. B) Novel protein coding and noncoding loci. Bar graph opacity refers to transcript type(protein coding, noncoding) C) Novel exon composition of novel isoforms, by isoform type. Labels indicate number of transcripts.

             Novel isoforms can occur due to an omission of a previously annotated exon, commonly referred as exon skipping or the addition of an unannotated exon which we refer to as a novel exon. We further classified novel exons by the biological process that may be driving their formation: alternative promoter usage driving the addition of novel first exons (FE), alternative polyadenylation driving the addition of novel terminal exons (TE), or alternative splicing driving the formation of all novel exons that are not the first or last exon [25], [26], [27]. We then split alternatively spliced exons into their commonly seen patterns, alternative 5’ splice site (A5SS), alternative 3’ splice site (A3SS), and retained introns (RI). Exons whose entire sequence was unannotated and is not a retained intron are fully novel exons. We note that all three of these mechanisms can lead to exon skipping, so for simplicity we grouped all novel isoforms resulting from exon skipping together. We found that the majority of novel exons within our dataset are novel FEs. We observed that the majority of RI exons lead to novel ORFs, whereas novel isoforms with omitted exons more often lead to noncoding isoforms. (Fig 3C)

## *De novo* transcriptomes match previously published experimental data better than existing annotation

             We validated *de novo* transcriptomes using three independent approaches. We first looked for evolutionary conservation of novel transcripts, since conservation is commonly accepted as a proxy for functional significance. We used the PhyloP 20 way species alignment, a measure of conservation between species, to calculate the average conservation score for each exon in the DNTX annotation and compared that to the average conservation score for each exon in the Gencode annotation [28]. We found that, on average, exons in the DNTX annotation are more conserved than exons in the Gencode annotation (pvalue <2.2e-16) (Supplemental Figure 2A).

             Next, since we observed an enrichment in novel first and last exons within our data set, we decided to evaluate the transcriptional start sites (TSS) and transcriptional end sites (TES) within the DNTX annotation by comparing it to two well-established and experimentally validated annotation databases: FANTOM and the polyA Atlas [29], [30]. We compared DNTX and Gencode TSS’s to CAGE-seq data from the FANTOM consortium; as CAGE-seq is optimized to detect the 5’ end of transcripts, we reasoned that it can serve as a valid ground truth set to evaluate TSS detection [31]. We calculated the absolute distance of TSS’s to CAGE peaks, and found that, on average, DNTX TSS’s were closer to CAGE peaks than Gencode TSS’s (pvalue <2.2e-16)(Supplemental Figure 2B).

             Finally, we evaluated TES’s using the polyA Atlas, which is comprised of polyadenylation signal annotation generated from aggregating 3’ seq data from multiple studies. As 3’-seq data is designed to accurately capture the 3’ ends of transcripts, it can similarly serve as a ground truth set to evaluate the accuracy of TES’s [32]. We calculated the absolute distance of DNTX TES’s to annotated polyA signals and compared them to the absolute distance of Gencode TES’s to polyA signals. We found that on average DNTX TES’s are closer to annotated polyadenylation signals than gencode TSS’s (pvalue <2.2e-16) (Supplemental Figure 2C)

## Novel Isoforms identified in ocular tissues

             Using the pan-eye transcriptome, we compared the overlap in constructed novel isoforms across ocular subtissues and found that 77.968 % of novel isoforms are specific to a singular ocular subtissue (Fig 4A). Additionally, fetal-like tissues had more novel isoforms than their adult counterpart. For each novel isoform we then calculated fraction isoform usage (FIU), or the fraction of total gene expression a transcript contributes to its parent gene. We found that, on average, novel isoforms contributed to 20.584 % of their parent gene’s expression but in each subtissue we found multiple novel isoforms that contribute to the majority of their parent genes expression (Fig 4B)

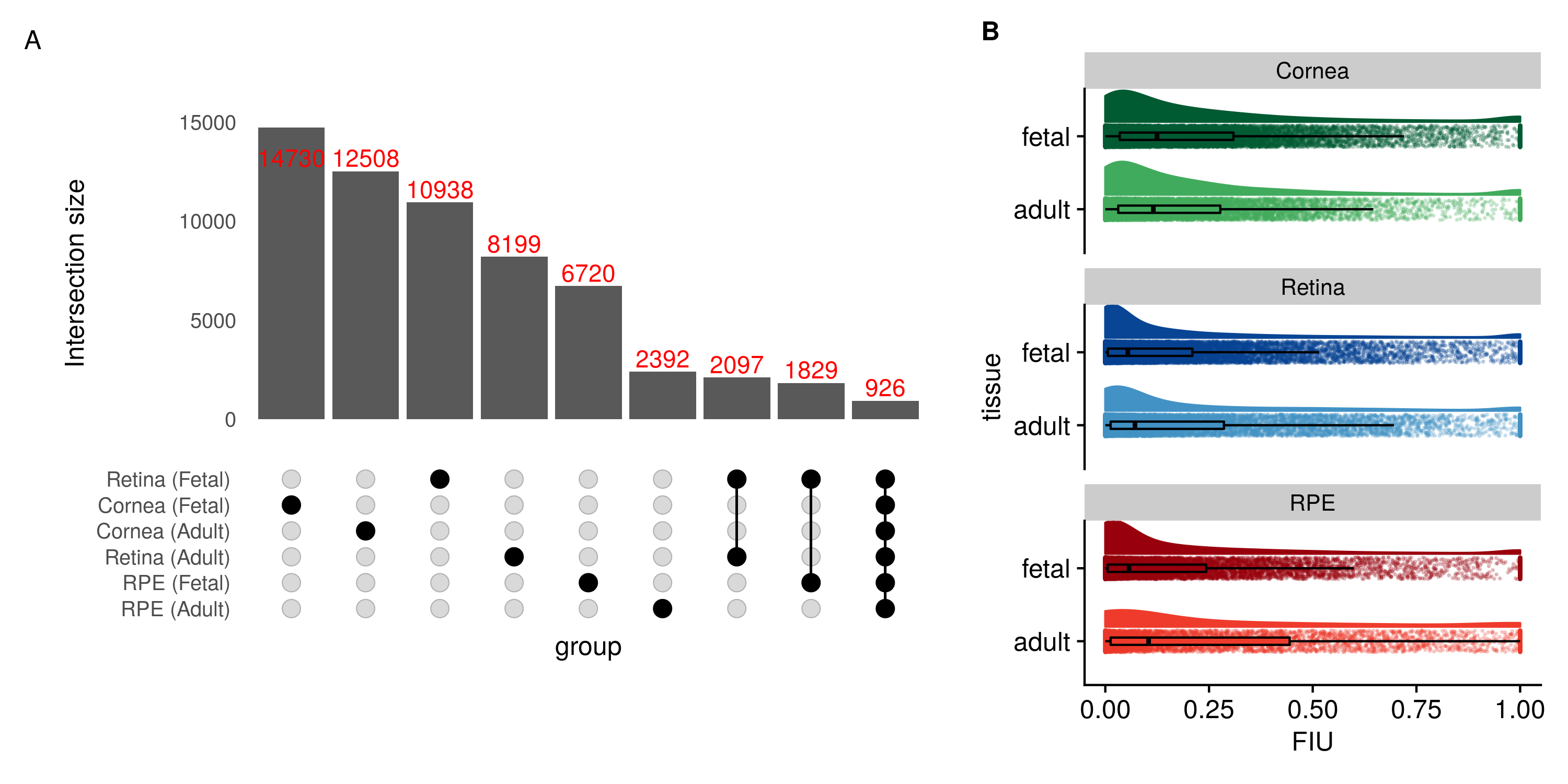


Figure 4. Overview of novel gene isoforms in the eye. A) Set intersection of novel isoforms in ocular transcriptomes. B) Boxplots of fraction isoform usage (FIU) overlaid over FIU data points with estimated distribution of data set above each boxplot.

## Differential usage of gene isoforms occurs during retinal development

             Multiple studies have shown that gene isoforms play a significant role in eye development [33], [34]. We hypothesized that the DNTX annotation could provide additional insight into alternative isoform usage and identify novel gene isoforms potentially involved in eye development. We used RNA-seq data of the developing retina from Mellough *et al*, an independent data set that we did not include for transcriptome construction, and used a subset of the DNTX annotation corresponding to fetal retina to quantify transcript expression. We then identified transcripts with significant changes in expression across retinal development (Figure 5). Transcripts that are differentially expressed (qvalue <.01) and have a mean FIU difference of .25 in at least one comparison of time points are indicative of differential transcript usage (DTU).

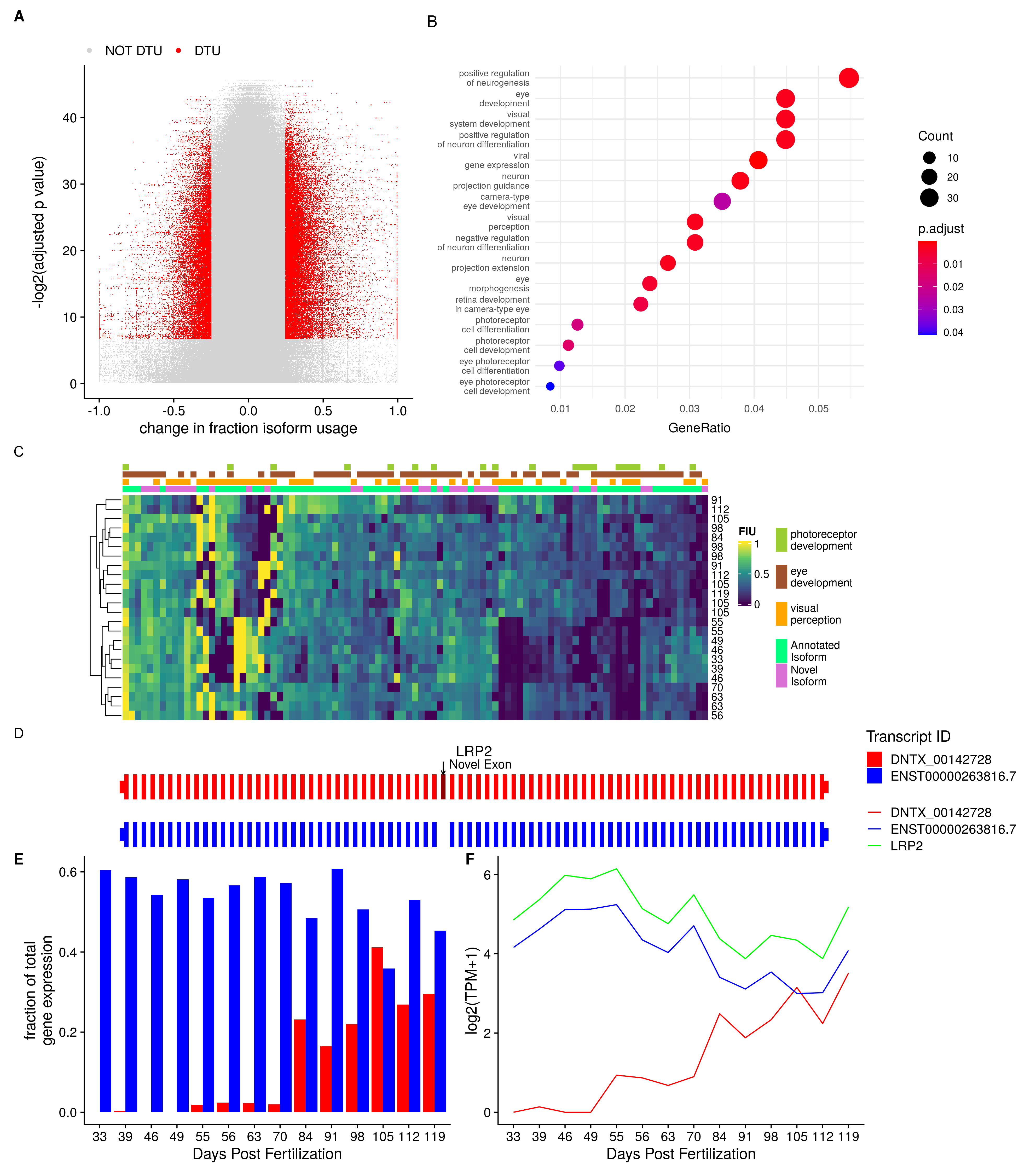


Figure 5 Differential transcript usage (DTU) during retinal development. A) Volcano plot of tested transcripts B) Dot plot for gene set enrichment analysis of significant DTU genes C) Heatmap of hierarchical clustering of transcripts with DTU associated with eye development D) Transcript models for *LRP2*, a gene undergoing DTU (not drawn to scale). Novel exon shaded dark red. E) FIU change in *LRP2* FIU across development F) average log-transformed TPM expression of *LRP2* isoforms across retinal development

             We analyzed the 24 Mellough *et al.* samples across 14 developmental days post fertilization and found 1717 transcripts across 812 genes displaying DTU (Fig 5A). We found that genes involved in DTU are enriched(qvalue <.05) for genes related to eye and neurological development (Fig 5B), and that hierarchical clustering of DTU transcripts generates an early stage and late stage cluster (Fig 5C). One of these genes, *LRP2* , is a classical example of DTU. *LRP2* is associated with the positive regulation of neurogenesis GO term and has been shown previously to play a role in retinal development [35]. Its novel isoform contains a single novel exon (exon 44) within its protein coding region (Fig 5D). *LRP2* is actively expressed throughout development; expression of its canonical isoform ENST00000263816.7 remains relatively unchanged across development, but its novel isoform DNTX\_00142728 shows a significant change in gene expression across development, accounting for nearly half of total *LRP2* expression at DNTX\_00142728’s peak expression(Fig 5E,F). A full list of genes and transcripts displaying DTU during retinal development is available in Supplemental Data (Supplemental Data 4).

             To confirm our computational analyses, we experimentally validated the novel *LRP2* isoform. As developing human retinal samples are difficult to obtain, we assayed *LRP2* expression via RT-PCR in iPSCs and iPSC-derived retinal organoids(see methods). As a control, we additionally assayed its expression in human fibroblasts. We designed primers targeting the novel exon in *LRP2* as well as primers targeting a canonical exon of the transcript. We confirm expression of both the novel and canonical exons in retinal organoids, and see no expression of either exon in fibroblasts (Supplemental Figure 3).

## *De novo* transcriptomes allow for a more precise variant prioritization.

             The identification of a disease-causing variant through genome sequencing is a common step in diagnosing genetic disease, when disease causing variants cannot be determined from exonic sequencing. Prediction of a variant’s biological impact and subsequent variant prioritization is a fundamental step in this process. Many methods for predicting variant effects on protein function or gene expression are based on location within the body of a transcript; for example variants that disrupt splice sites and start/stop codons are considered to be the most damaging, while variants within intronic and intergenic regions have unknown impact or are not classified, and, thus, are not included for further consideration. However, multiple studies have identified pathogenic deep intronic variants for retinal dystrophies [36], [37], [38], [39], [40], [41], [42]. Pathogenic intronic variants are thought to function by introducing a novel splice site, disrupting regulatory motifs, or altering a tissue-specific transcript. To explore this third possibility, we mapped known pathogenic intronic variants onto novel isoforms within the *de novo* transcriptomes.

             We used a list of 129 intronic and noncoding variants previously identified as pathogenic for a retinal dystrophy and predicted the effect of these variants with Ensembl’s Variant Effect Predictor using a subset of the DNTX annotation corresponding to fetal and adult retina as the input transcript annotation. We identified ten variants whose predicted effect increased in severity due the presence of a novel gene isoform in a previously intronic region (Table 2). Seven of these variants were in deep intronic hotspots known for pathogenic variation within the gene *ABCA4*.

| **Gene Name** | **Associated Disease** | **Location (hg19)** | **Canonical Variant HGVS** | **Gencode Predicted Consequence** | **DNTX Predicted Consequence** | **Published Study** |
| --- | --- | --- | --- | --- | --- | --- |
| ABCA4 | ABCA4-associated maculopathy | Chr1:94481967 C>T | c.5197–557G>T, NM\_000350.2 | intron variant, downstream gene variant | 5 prime UTR variant | Bauwens et al. |
| Chr1:94546814 G>C | c.859–540C>G, NM\_000350.2 | intron variant | non coding transcript exon variant |
| Stargardt disease | Chr1:94484001 C>T | c.5196+1137G>A, NM\_000350.2 | intron variant, downstream gene variant | 5 prime UTR variant | Braun et al. Zernant et al. |
| Chr1:94484082 T>G | c.5196+1056A>G, NM\_000350.2 | intron variant, downstream gene variant | 5 prime UTR variant |
| Chr1:94526934 T>G | c.1938-619A>G, NM\_000350.2 | intron variant, splice region variant, non coding transcript variant | non coding transcript exon variant | Zernant et al. |
| Chr1:94527698 G>C | c.1937+435C>G, NM\_000350.2 | intron variant, upstream gene variant | non coding transcript exon variant | Sangermano et al. |
| Chr1:94546780 C>G | c.859-506G>C, NM\_000350.2 | intron variant | non coding transcript exon variant |
| IFT140 | Ciliopathy | Chr16:1576595 C>A | c.2577+25G>A, NM\_014714.3 | upstream gene variant, intron variant, NMD transcript variant, non coding transcript exon variant, non coding transcript variant | missense variant | Geoffroy et al. |
| PROM1 | Cone–rod dystrophy | Chr4:15989860 T>G | c.2077-521A>G, NM\_006017.2 | intron variant, upstream gene variant | 5 prime UTR variant | Mayer et al. |
| RPGRIP1 | RPGRIP1-mediated inherited retinal degeneration | Chr14:21789588 G>A | c.1611+27G>A, NM\_020366.3 | intron variant, non coding transcript variant, upstream gene variant, synonymous variant, NMD transcript variant, downstream gene variant | 5 prime UTR variant | Jamshidi et al. |

Table 2. Pathogenic variants previously considered intronic that are on expressed transcripts in the retina *de novo* transcriptome. Canonical human genome variation society (HGVS) annotation is based on transcripts from the RefSeq annnotation. Predicted consequences were generated with the Variant Effect Predictor(VEP)

             These variants were spanned by three distinct novel isoforms with two containing open reading frames (ORFs) encoding only the carboxy-terminus of the canonical protein isoform, and one noncoding spanning the proximal half of the canonical isoform (Fig 6). *ABCA4* expression and function has also been observed in RPE [43]. However, we did not observe these transcripts in RPE, suggesting that these pathogenic variants are primarily affecting retinal-specific *ABCA4* transcripts. We note that these transcripts have not been experimentally validated.

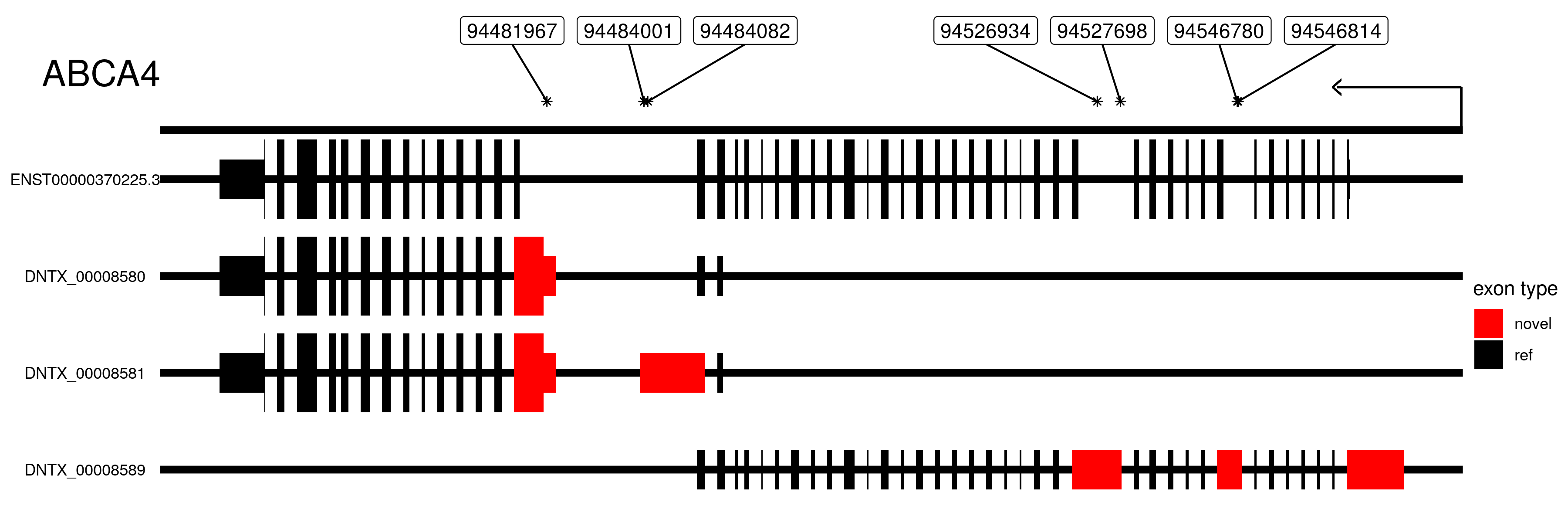


Figure 6. Transcript models for selected isoforms of *ABCA4* along with location of pathogenic intronic variants. Location is on the hg19 human genome build. Thick lines indicate protein coding regions. Arrow indicates direction of transcription. Introns not drawn to scale

             To further highlight the potential importance of *de novo* transcriptomes for future genetic tests we determined how many genes associated with retinal disease from RetNet (sph.uth.edu/retnet/) have novel isoforms. We found that within the set of genes with novel isoforms, there is significant enrichment of retinal disease genes (hypergeometric pvalue = 3.4e-04), with 220 out of 379 RetNet genes having a novel isoform. A full list of these genes is available in the Supplementary data(supplemental data 5).

## A companion visualization tool enables easy use of *de novo* transcriptomes

             To make our results easily accessible we designed a R-Shiny app for visualizing and accessing our *de novo* transcriptomes. For each subtissue we show the FIU for each transcript associated with a gene (Fig 7A). We show the exon-intron structure of each transcript and mousing over exons show genomic location overlapping SNPs, and phylogenetic conservation scores (Fig 7B). We additionally show a barplot of the fraction of samples each transcript was constructed in (Fig 7C). Users can also download the *de novo* transcriptomes for selected subtissues in GTF and fasta format. Instructions to download and run the app are available at <https://github.com/vinay-swamy/ocular_transcriptomes_shiny>. While visualization of direct transcript expression is not a part of this app, it can be viewed in the eyeIntegration app by selected ‘DNTX’ as the transcript annotation [16]. Finally, we provide all code as a Snakemake workflow and provide a Docker container with all software required for the pipeline available at <https://github.com/vinay-swamy/ocular_transcriptomes_pipeline>

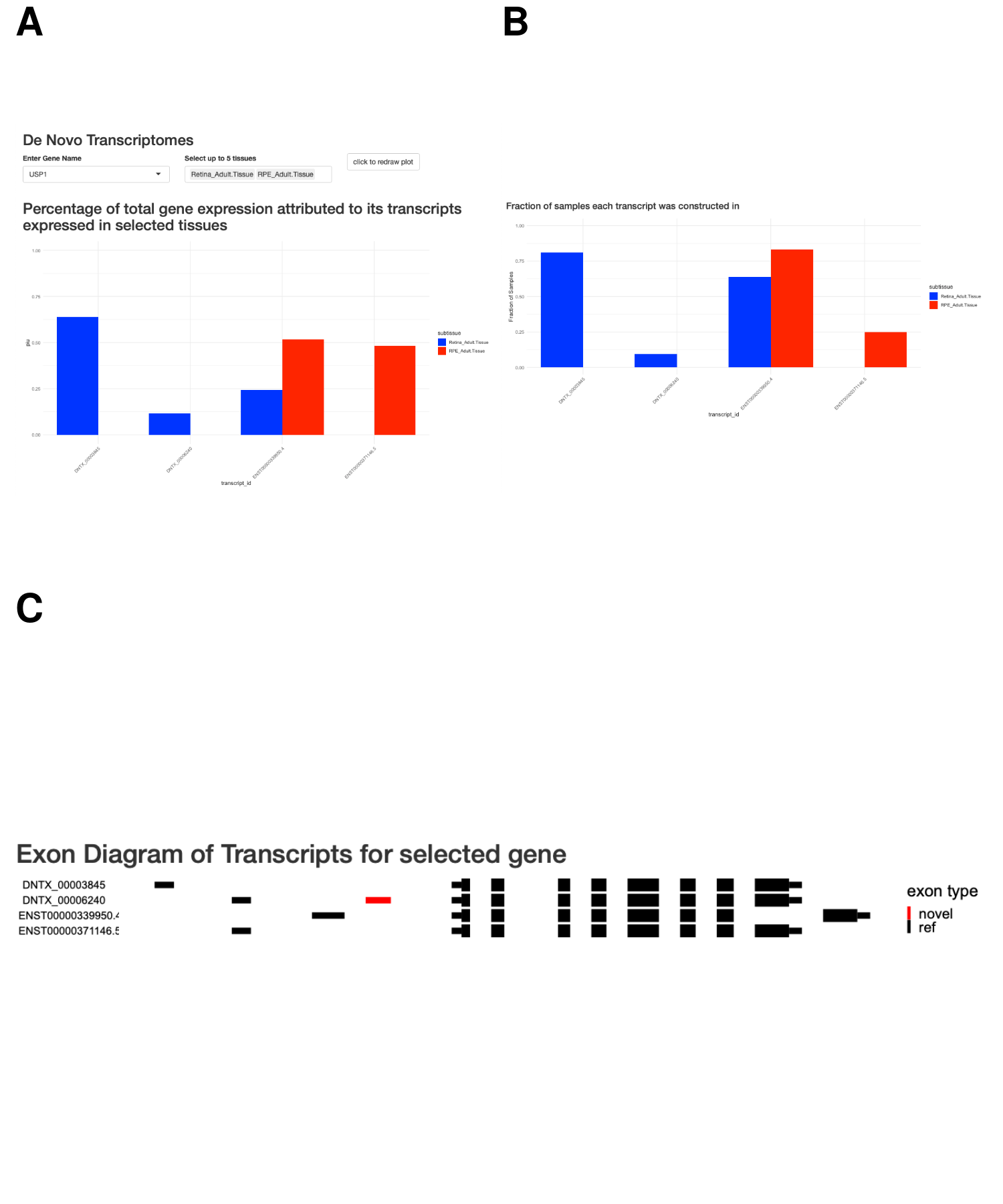


Figure 7. Screenshots from dynamic *de novo* transcriptome visualization tool. A). FIU bar plot for selected gene and subtissue. B). Exon level diagram of transcript body. Thicklines represent coding region of transcript. Novel exons colored in red. Tooltip contains genomic location and phylop score for each exon C) Bargraph of fraction of samples within dataset each transcript was consructed in by tissue.

# Discussion

             Motivated by the lack of a comprehensive transcriptome for the eye, we constructed transcriptomes for adult and fetal retina, RPE and cornea. By using long-read RNA-seq data to calibrate our short-read construction pipeline, we were able to identify biologically relevant transcriptomes. We found that concordance between long and short-read-based transcriptomes is directly related to transcript length and transcript expression. We saw a clear inability within the PacBio data set to accurately detect transcripts shorter than 2000bp for both previously annotated and novel transcripts. As many of the transcripts constructed using short-reads are below this threshold, long-read sequencing data enriched for smaller transcript sizes would provide greater insight in future studies.

             We used a large dataset compiled from published RNA-seq data to build the pan-eye transcriptomes, an approach that has several key advantages. First, the large sample size overcomes the noisy nature of RNA-seq data. Second, as the cohort is constructed from many independent studies, we are more confident that the transcriptomes accurately reflect the biology of their originating subtissue and are not a technical artifact due to preparation of the samples. As another line of evidence, the *de novo* transcriptomes match existing large scale data sets and are more conserved than existing annotations (Supplemental Figure 2).

             In each ocular subtissue we examined, we found hundreds of novel gene isoforms, many of which were novel due to novel exons. Within ocular subtissues, these novel isoforms are most commonly specific to single subtissue. This makes sense as a majority of the exons in our *de novo* transcriptomes are first and last exons, which have been previously shown to significantly contribute to the tissue specificity of gene isoforms [44]. We also found that on average novel isoforms represent about 20.584 % of their parent gene’s expression. Future studies are needed to identify the function of these isoforms. One possibility is that some of these isoforms are only expressed in rare cell types, as transcript annotation was previously shown to be incomplete in rare cell types [9]. This especially makes sense in the retina which contains over a dozen distinct cell types, several of which contribute to 5% or less of the total cell population [45]. As we imposed a strict expression filter as part of our transcriptome pipeline, we may have removed transcripts specific to rare cell types.

             In conclusion, we created the first pan-eye transcriptome annotation and showed that it is useful in understanding the role of gene isoforms in ocular biology and improving the ability to diagnose inherited eye diseases. To make the transcriptomes easily accessible to other researchers we designed a webapp both for visualization and to quickly access tissue-specific annotation files. We believe this project will enable other researchers to explore new research directions and answer long pending questions.

# Materials and Methods

## Generation of PacBio long-read and Illumina short-read RNA sequencing data

             Human iPSCs were differentiated into RPE using previously described protocols [46], [47]. iPSC-derived RPE (iPSC-RPE) cells at 42 days post differentiation were lysed with TRIzol reagent (Thermo Fisher Scientific; cat # 15596026) and total RNA was isolated using the Direct-zol RNA MiniPrep Kit (Zymo Research, Irvine, CA). 5-6 µg total RNA that passed quality control metric (RIN >.9) were used for PacBio library preparation. For PacBio HiFi circular consensus sequencing(CCS), libraries were prepared following the “Procedure-Checklist-Iso-Seq-Express-Template-Preparation-for-Sequel-and-Sequel-II-Systems” protocol. Two libraries were generated: one to capture transcripts 2 kilobases(kb) or smaller, and one to capture transcripts between 2-5kb. Sequencing was done on the PacBio Sequel II system for a movie time of 24 hours.

             For Illumina sequencing, Poly-A selected stranded mRNA libraries were constructed from 0.5-1 µg total RNA using the Illumina TruSeq Stranded mRNA Sample Prep Kits according to manufacturer’s instructions. Amplification was performed using 10-12 cycles to minimize the risk of over-amplification. Unique dual-indexed barcode adapters were applied to each library. Libraries were pooled in equimolar ratio and sequenced together on a HiSeq 4000. At least 57 million 75-base read pairs were generated for each individual library. Data was processed using illumina Real Time Analysis (RTA) version 2.7.7. All library preparation and sequencing was performed at the National Institutes of Health Intramural Sequencing Center (NISC).

## Differentiation of human induced pluripotent stem cells (iPSC) into retinal organoids

             Retinal organoids were generated from 3D1 iPSC according to the protocols described previously [48], [49]. Briefly, embryoid bodies were generated by culturing 70 - 80% confluent 3D1 cells in suspension in Neural Induction Medium (NIM) for 7 days. After 7 days of differentiation, cell aggregates (embryoid bodies) were replated on matrigel (Corning; cat# 354277) coated plates and maintained in NIM. Then, the medium was switched from NIM to Retinal Induction Media (RIM) from days 16 - 27 of differentiation. On day 28 of differentiation, the aggregates that formed neurospheres were mechanically lifted and replated in suspension using Retinal Induction Medium(RIM). The cells were maintained in RIM through differentiation day 41. Next, RIM was replaced with Retina Differentiation Medium (RDM) from days 42 – 60 of differentiation. Lastly, retinal organoids were mechanically dissected out on day 60 of differentiation, transferred to ultra-low adhesion dishes and maintained in suspension in RDM. Media compositions are as follows: NIM: 489.5 mL 1:1 ratio DMEM (ThermoFisher, cat#11995073) and F12 (ThermoFisher, cat# 1765054), 5 mL CTS N2 supplement (ThermoFisher, cat# 17502048), 5 mL MEM NEAA (MilliporeSigma, cat# M7145), 0.5 mL 2mg/ml heparin (Sigma, cat# H3393); RIM: 240 mL 1:1 ratio DMEM and F12, 240 mL DMEM, 10 mL B27 without Vitamin A (ThermoFisher, cat# 12587010), 5 mL MEM NEAA, 5 mL penstrep (ThermoFisher, cat# 15070063), 500 uL 1000x Fungin (Invivogen, cat# ANTFN1 ANTFN2); RDM: 240 mL DMEM/F12, 240 mL DMEM, 50 mL FBS (ThermoFisher, cat# 16000069), 10 mL B27 without Vitamin A, 5 mL MEM NEAA, 5 mL GlutaMAX (ThermoFisher, cat# 35050061), 200 uL IGF-1 (ThermoFisher, cat# PHG0071), 5 mL 100mM Taurine (MilliporeSigma, cat#T5691), 5 mL penstrep, 500 uL 1000x Fungin.

## RNA extraction and experimental confirmation of retina-specific exons using RT-PCR

             Ten high-quality retinal organoids at day 200 of differentiation were pooled and total RNA was extracted using the TRIzol reagent (Thermo Fisher Scientific, cat # 15596026) and the Direct-zol™ RNA Miniprep Kit (Zymo Research, Irvine, CA). Retinal organoids generated from two independent differentiations were used for RNA extraction. One microgram total RNA was reverse-transcribed into cDNA using SuperScript IV RT Kit with OligodT (Thermo, Catalog number: 12594025). PCR primers for the target exons were designed using the Primer-BLAST program from NCBI and RT-PCR assays were performed on Veriti 96-Well Thermal Cycler (Thermo Fisher Scientific) using Taq DNA polymerase (Thermo Scientific) with the following conditions: initial denaturation at 94 °C for 2 min followed by 425 cycles of 94 °C for 1 minute, 60 °C for 1 minute, 72 °C for 2 minutes, and a final extension at 72 °C for 5 min. The PCR products were subsequently resolved on 2% agarose gel using electrophoresis. LRP2 RT-PCR primers are: Exon 2/3 Forward: TTGGGGATGCATTGTCCCTC; Exon 2/3: Reverse: AGACTGTTCAGATGACGCGG; Exon43/nvl\_exon120703 Forward: AACGCTGCAAAATGGAC; Exon43/nvl\_exon120703 Reverse: TGGTGAACGATGTGGTGC

## Analysis of long-read data

             PacBio sequencing movies were processed into full length, non-chimeric (FLNC) reads using the IsoSeq3 3.1.2 pipeline in the PacBio SMRT link v7.0 software. The existing ENCODE long-read RNA-seq pipeline (<https://github.com/ENCODE-DCC/long-read-rna-pipeline>) was rewritten as a Snakemake workflow as follows. Transcripts were aligned to the human genome using minimap2(18), using an alignment index built on the gencode v28 primary human genome. Sequencing errors in aligned long-reads were corrected using TranscriptClean (19) with default parameters. Splice junctions for TranscriptClean were obtained using the TranscriptClean accessory script “get\_SJs\_from\_gtf.py” using the gencode v28 comprehensive transcript annotation as the input. A list of common variants to avoid correcting were obtained from the ENCODE portal (<https://www.encodeproject.org/files/ENCFF911UGW/>). The long-read transcriptome annotation was generated with TALON (20). A TALON database was generated using the talon\_initialize\_database command, with all default parameters, except for the “–5P” and “–3p” parameters. These parameters represent the maximum distance between close 5’ start and 3’ ends of similar transcript to merge and were both set to 100 to match parameters used in later tools. Annotation in GTF format was generated using the talon\_create\_GTF command, and transcript abundance values were generated using the talon\_abundance command.

## Analysis of short-read RPE data

             Each sample was aligned to the Gencode release 28 hg38 human genome assembly using the genomic aligner STAR and the resulting BAM files were sorted using samtools sort [8],[50],[51]. For each sorted BAM file, a per-sample base transcriptome was constructed using StringTie with the Gencode v28 comprehensive annotation as a guiding annotation [8],[12]. All sample transcriptomes were merged with the long-read transcriptome using gffcompare with default parameters [52]. We note that the default values for the distance to merge similar 5’ starts and 3 ends of transcripts in gffcompare is the same to what we chose for TALON. We defined the metric construction accuracy, used to evaluate short-read transcriptome construction as the following:

## Construction of subtissue-specific transcriptomes.

            We constructed transcriptomes for 1217 samples in the Eye in a Disk(EiaD), a dataset generated from aggregating publically available healthy, unperturbed RNA-seq samples from 50 distinct locations of the body across 29 different studies. Specific information on how this dataset was generated is detailed in the methods from our previous work [16]. We constructed a transcriptome for each sample, and merged samples together to create 50 subtissue-specific transcriptomes. We define subtissue as a unique body location and are either temporally different versions of the same tissue(adult vs fetal tissue), or different regions of a larger tissue (cortex vs cerebellum in brain). Tissue refers to complete whole tissue (retina, brain, liver). For each subtissue-specific transcriptome, we removed transcripts that had an average expression less than 1 Transcripts Per Million (TPM) across all samples of the same subtissue type. All subtissue-specific transcriptomes were merged to form a single unified annotation file in general transfer format(GTF) to ensure transcript identifiers were the same across subtissues. We merged all ocular subtissue transcriptomes to generate a separate pan-eye transcriptome.

## Subtissue specific transcriptome quantification

             For each resulting subtissue specific transcriptome, we extracted transcript sequences using the tool gffread and used these sequences to build a subtissue-specific quantification index using the index mode of the alignment-free quantification tool Salmon [52], [53]. For each sample, we quantified transcript expression using the quant mode of Salmon, using a sample’s respective subtissue specific quantification index. We similarly quantified all ocular samples using the pan-eye transcriptome and the Gencode v28 reference transcriptome.

## Annotation of novel exons

             First, a comprehensive set of distinct, annotated exons was generated by merging exon annotation from gencode, ensembl, UCSC, and refseq. We then defined a novel exon as any exon within our transcriptomes that does not exactly match the chromosome, start, end and strand of an annotated exon. Novels exons were classified by splitting exons into 3 categories: first, last, and middle exons. We then extracted all annotated exon start and stop sites from our set of previously annotated exons. Novel middle exons that have an annotated start but an unannotated end were categorized as a novel alternative 3’ end exons and similarly novel middle exons with an unannotated start but annotated end were categorized as a novel alternative 5’ start exons. Novel middle exons whose start and end match annotated exon start and ends were considered retained introns. Novel middle exons whose start and end do not match annotated starts and ends were considered fully novel exons. We then classified novel first and last exons. Novel first exons were first exons whose start is not in the set of annotated exon starts, and novel last exons were terminal exons whose end is not in the set of annotated exon ends. This analysis of novel transcripts is implemented in our Rscript “annotate\_and\_make\_tissue\_gtfs.R”.

## Validation of DNTX with phylop, CAGE data, and polyA signals

             PhyloP scores for the phylop 20-way multi species alignment were downloaded from UCSC’s FTP server on October 16th, 2019 and converted from bigWig format to bed format using the wig2bed tool in BEDOPs [28], [54]. The average score per exon in both the gencode and DNTX annotation was calculated by intersecting exon locations with phylop scores and then averaging the per base score for each exon, using the intersect and groupby tools from the bedtools suite, respectively. Significant difference in mean phylop score was tested with a Mann Whitney U test.

             CAGE peaks were download from the FANTOM FTP server (<https://fantom.gsc.riken.jp/5/datafiles/reprocessed/hg38_latest/extra/CAGE_peaks/hg38_fair+new_CAGE_peaks_phase1and2.bed.gz>) on June 15th 2020 [29]. Transcriptional start sites (TSS) were extracted from gencode and DNTX annotations; TSS is defined as the start of the first exon of a transcript. Distance to CAGE peaks was calculated using the closest tool in the bedtools suite. Significant difference in mean distance to CAGE peak between DNTX and gencode annotation was tested with a Mann Whitney U test.

             Polyadenylation signal annotations were downloaded from the polyA site atlas (<https://polyasite.unibas.ch/download/atlas/2.0/GRCh38.96/atlas.clusters.2.0.GRCh38.96.bed.gz>) on June 15th 2020 [30]. Transcriptional end sites(TES) were extracted from gencode and DNTX annotations; TES is defined as the end of the terminal exon of a transcript. Distance to polyA signal was calculated using the closest tool in the bedtools suite [55]. Significant difference in mean distance to polyA signal was tested with a Mann Whitney U test.

## Identification of novel protein coding transcripts

             Protein-coding transcripts in the unified transcriptome were identified using the TransDecoder suite [11]. Transcript sequences in fasta format were extracted from the final pan-body transcriptome using the TransDecoder util script “gtf\_genome\_to\_cdna\_fasta.pl”. Potential open reading frames(ORFs) were generated from transcript sequences using the LongestORF module within TransDecoder, and the single best ORF for each transcript was extracted with the Predict module within Transdecoder. The resulting ORFs were mapped to genomic locations with the TransDecoder util script “gtf\_to\_alignment\_gff3.pl”. For each ORF start and stop codons were extracted with the script “agat\_sp\_add\_start\_stop.pl” scripts from the AGAT toolkit (<https://github.com/NBISweden/AGAT/>). Transcripts with no detectable ORF or missing a start or stop codon were labelled as noncoding.

## Analysis of novel isoforms in eye tissues

             An Upset plot was generated using the ComplexUpset package (<https://github.com/krassowski/complex-upset>) [56]. Fraction Isoform Usage (FIU) was calculated for each transcript *t* associated with a parent gene *g* using the following formula: . Raincloud plots of FIU were generated using the “R\_Rainclouds” R package [57].

## Analysis of fetal retina RNA-seq data.

             RNA-seq samples from Mellough *et al*. were obtained from EiaD, and were not included in the main dataset used for building transcriptomes. Outliers within the dataset were identified by first performing principal component analysis of transcript level expression data, calculating the center of all data using the first two principal components, and subsequently removing five samples furthest away from the center of all data. The remaining samples were normalized using calcNormFactors from the R package edgeR and converted to weights using the voom function from the R package limma [58], [59]. Differential expression was modeled using the lmFit function using developmental time point as the model design and tested for significant change in expression using the Ebayes function from limma. Gene Set enrichment was tested using the R package clusterprofileR [60]. Heatmaps were generated using the ComplexHeatmap package [61].

## Prediction of variant impact using *de novo* transcriptomes.

             Noncoding variants previously associated with retinal disease from the Blueprint Genetics Retinal dystrophy panel were obtained from the Blueprint Genetics website (<https://blueprintgenetics.com/tests/panels/ophthalmology/retinal-dystrophy-panel/>). The variants were converted from HGVS to VCF format using a custom python script “HGVS\_to\_VCF.py”. This VCF was then remapped to the hg38 human genome build using the tool crossmap [62]. The VCF of variants was used as the input variants for the Variant Effect Predictor(VEP) tool from Ensembl, with each subtissue specific transcriptome as the input annotation [63]. VEP was additionally run using the gencode v28 comprehensive annotation as the input annotation to identify variants whose predicted impact increased in severity.

## Figures, Tables, and Computing Resources

             All statistical analyses, figures and tables in this paper were generated using the R programming language [64]. A full list of packages and versions can be found in the supplementary file session\_info.txt. The cartoon diagram of iPSC-derived retinal pigment epithelium cells (figure 1) was created with [BioRender.com](https://biorender.com). All computation was performed on the National Institutes of Health high performance computer system Biowulf (hpc.nih.gov).

## Code and data availability

             To improve reproducibility, all code used for both analyzing the data and generating the figures for this paper was written as multiple Snakemake pipelines. Each Snakefile contains the exact parameters for all tools and scripts used in each analysis [20]. All code (and versions) used for this project is publicly available in the following github repositories: <https://github.com/vinay-swamy/ocular_transcriptomes_pipeline> (short read *de novo* transcriptome pipeline pipeline), <https://github.com/vinay-swamy/ocular_transcriptomes_longread_analysis> (long-read analysis pipeline), <https://github.com/vinay-swamy/ocular_transcriptomes_paper> (figures and tables for this paper), <https://github.com/vinay-swamy/ocular_transcriptomes_shiny> (visualization and gtf download webapp). Additionally, all Snakefiles are included as supplementary data.(supplementary data files 1-3)

             All sequencing data generated by this project is available through the BioProject portal: <http://www.ncbi.nlm.nih.gov/bioproject/726583> ( PRJNA726583). The accessions of all public data used by are available on the Sequence Read Archive, and we provide a table of relevant accessions in the supplemental data (supplemental data 6). Additionally, annotation files in GTF format for the DNTX and pan-eye annotation are includded in the supplemental data/ (supplemental data 7,8)

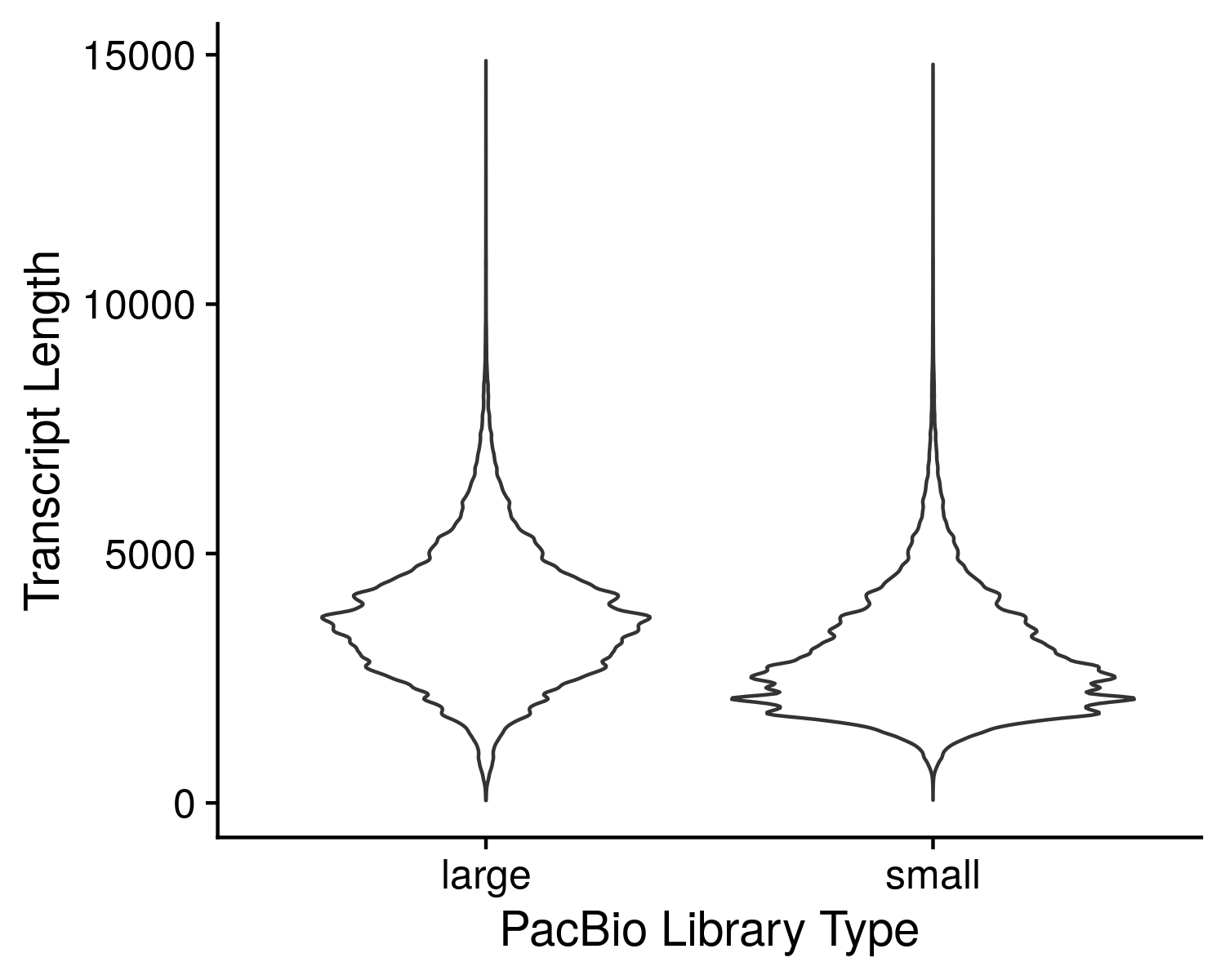
# Author Contributions

VSS and DMM designed overall study. VSS implemented all computational pipelines and analyses. TGF and AM conducted all bench experiments. VSS, DM, TGF, and RBH drafted and edited this manuscript.

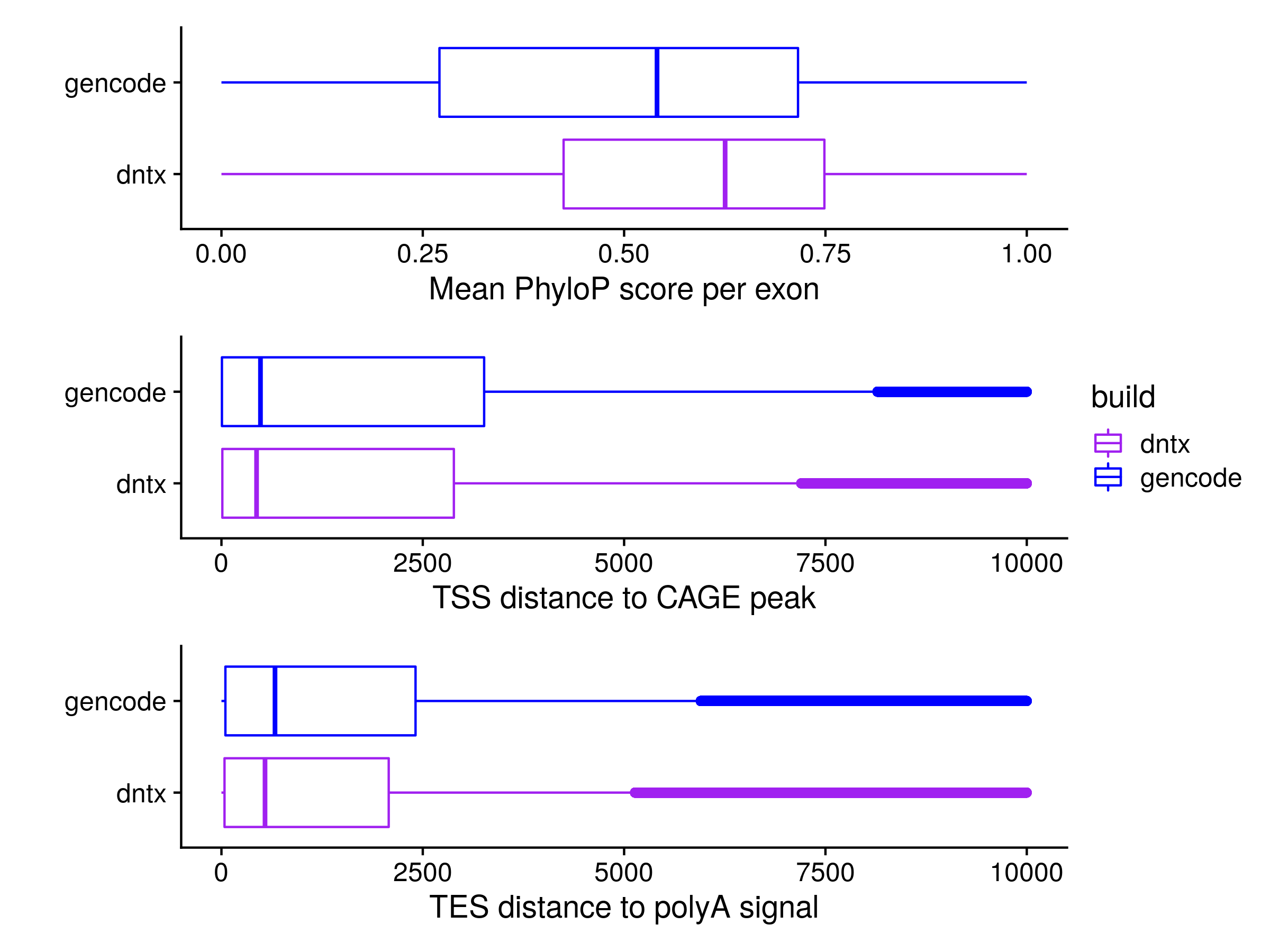
# Competing Interests

             All authors declare no competing interests.

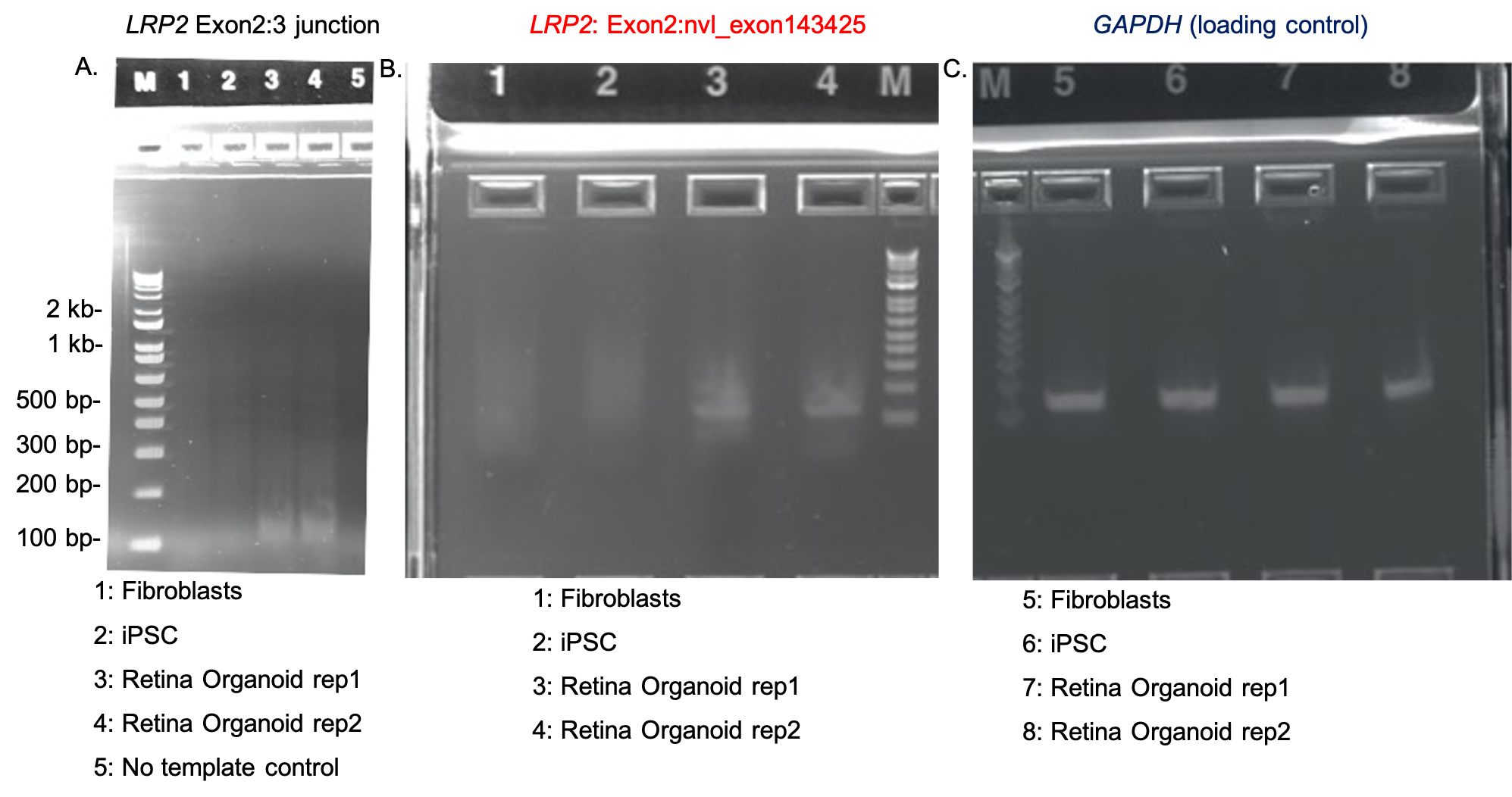
# Supplemental Figures



Supplemental Figure 1. Distribution of PacBio long-read lengths for two library sizes.



Supplemental Figure 2. Comparison of DNTX annotation to Gencode annotation. A) Average per exon Phylop score for Gencode and DNTX transcripts. B) Average distance of DNTX transcriptional start sites (TSS) and Gencode TSS to CAGE-seq peaks from the FANTOM consortium. C) Average distance of DNTX transcriptional end sites (TES) and Gencode TES to polyadenylation signals in the PolyA site atlas.



Supplemental Figure 3. PCR amplification of LRP2 Transcript in fibroblasts, iPSCs, and Retinal Organoids. A) Amplification of canonical exon 2:3 junction (control) B) Amplification of Novel Exon 44 C) GAPDH loading control

# References

1. Dykes IM, Bueren KL van, Scambler PJ. HIC2 regulates isoform switching during maturation of the cardiovascular system. Journal of Molecular and Cellular Cardiology. 2018;114: 29–37. doi:[10.1016/j.yjmcc.2017.10.007](https://doi.org/10.1016/j.yjmcc.2017.10.007)

2. Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, Baren MJ van, et al. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nature Biotechnology. 2010;28: 511–515. doi:[10.1038/nbt.1621](https://doi.org/10.1038/nbt.1621)

3. Mitra M, Lee HN, Coller HA. Splicing Busts a Move: Isoform Switching Regulates Migration. Trends in Cell Biology. 2020;30: 74–85. doi:[10.1016/j.tcb.2019.10.007](https://doi.org/10.1016/j.tcb.2019.10.007)

4. Vitting-Seerup K, Sandelin A. The Landscape of Isoform Switches in Human Cancers. Molecular Cancer Research. 2017;15: 1206–1220. doi:[10.1158/1541-7786.MCR-16-0459](https://doi.org/10.1158/1541-7786.MCR-16-0459)

5. Neagoe Ciprian, Kulke Michael, del Monte Federica, Gwathmey Judith K., de Tombe Pieter P., Hajjar Roger J., et al. Titin Isoform Switch in Ischemic Human Heart Disease. Circulation. 2002;106: 1333–1341. doi:[10.1161/01.CIR.0000029803.93022.93](https://doi.org/10.1161/01.CIR.0000029803.93022.93)

6. Mills JD, Nalpathamkalam T, Jacobs HIL, Janitz C, Merico D, Hu P, et al. RNA-Seq analysis of the parietal cortex in Alzheimer’s disease reveals alternatively spliced isoforms related to lipid metabolism. Neuroscience Letters. 2013;536: 90–95. doi:[10.1016/j.neulet.2012.12.042](https://doi.org/10.1016/j.neulet.2012.12.042)

7. Perrin RM, Konopatskaya O, Qiu Y, Harper S, Bates DO, Churchill AJ. Diabetic retinopathy is associated with a switch in splicing from anti- to pro-angiogenic isoforms of vascular endothelial growth factor. Diabetologia. 2005;48: 2422–2427. doi:[10.1007/s00125-005-1951-8](https://doi.org/10.1007/s00125-005-1951-8)

8. Frankish A, Diekhans M, Ferreira A-M, Johnson R, Jungreis I, Loveland J, et al. GENCODE reference annotation for the human and mouse genomes. Nucleic acids research. 2019;47: D766–D773. doi:[10.1093/nar/gky955](https://doi.org/10.1093/nar/gky955)

9. Zhang D, Guelfi S, Garcia-Ruiz S, Costa B, Reynolds RH, D’Sa K, et al. Incomplete annotation has a disproportionate impact on our understanding of Mendelian and complex neurogenetic disorders. Science Advances. 2020;6: eaay8299. doi:[10.1126/sciadv.aay8299](https://doi.org/10.1126/sciadv.aay8299)

10. Nagalakshmi U, Wang Z, Waern K, Shou C, Raha D, Gerstein M, et al. The Transcriptional Landscape of the Yeast Genome Defined by RNA Sequencing. Science. 2008;320: 1344–1349. doi:[10.1126/science.1158441](https://doi.org/10.1126/science.1158441)

11. Haas BJ, Papanicolaou A, Yassour M, Grabherr M, Blood PD, Bowden J, et al. De novo transcript sequence reconstruction from RNA-Seq: Reference generation and analysis with Trinity. Nature protocols. 2013;8. doi:[10.1038/nprot.2013.084](https://doi.org/10.1038/nprot.2013.084)

12. Pertea M, Pertea GM, Antonescu CM, Chang T-C, Mendell JT, Salzberg SL. StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. Nature Biotechnology. 2015;33: 290–295. doi:[10.1038/nbt.3122](https://doi.org/10.1038/nbt.3122)

13. GTEx Consortium, Laboratory, Data Analysis &Coordinating Center (LDACC)—Analysis Working Group, Statistical Methods groups—Analysis Working Group, Enhancing GTEx (eGTEx) groups, NIH Common Fund, NIH/NCI, et al. Genetic effects on gene expression across human tissues. Nature. 2017;550: 204–213. doi:[10.1038/nature24277](https://doi.org/10.1038/nature24277)

14. Pertea M, Shumate A, Pertea G, Varabyou A, Breitwieser FP, Chang Y-C, et al. CHESS: A new human gene catalog curated from thousands of large-scale RNA sequencing experiments reveals extensive transcriptional noise. Genome Biology. 2018;19: 208. doi:[10.1186/s13059-018-1590-2](https://doi.org/10.1186/s13059-018-1590-2)

15. Wenger AM, Peluso P, Rowell WJ, Chang P-C, Hall RJ, Concepcion GT, et al. Accurate circular consensus long-read sequencing improves variant detection and assembly of a human genome. Nature Biotechnology. 2019;37: 1155–1162. doi:[10.1038/s41587-019-0217-9](https://doi.org/10.1038/s41587-019-0217-9)

16. Swamy V, McGaughey D. Eye in a Disk: eyeIntegration Human Pan-Eye and Body Transcriptome Database Version 1.0. Investigative Ophthalmology & Visual Science. 2019;60: 3236–3246. doi:[10.1167/iovs.19-27106](https://doi.org/10.1167/iovs.19-27106)

17. Blenkinsop TA, Saini JS, Maminishkis A, Bharti K, Wan Q, Banzon T, et al. Human Adult Retinal Pigment Epithelial Stem Cell-Derived RPE Monolayers Exhibit Key Physiological Characteristics of Native Tissue. Investigative Ophthalmology & Visual Science. 2015;56: 7085–7099. doi:[10.1167/iovs.14-16246](https://doi.org/10.1167/iovs.14-16246)

18. Maruotti J, Sripathi SR, Bharti K, Fuller J, Wahlin KJ, Ranganathan V, et al. Small-molecule–directed, efficient generation of retinal pigment epithelium from human pluripotent stem cells. Proceedings of the National Academy of Sciences. 2015;112: 10950–10955. doi:[10.1073/pnas.1422818112](https://doi.org/10.1073/pnas.1422818112)

19. Pertea M, Kim D, Pertea GM, Leek JT, Salzberg SL. Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown. Nature Protocols. 2016;11: 1650–1667. doi:[10.1038/nprot.2016.095](https://doi.org/10.1038/nprot.2016.095)

20. Köster J, Rahmann S. Snakemake—a scalable bioinformatics workflow engine. Bioinformatics. 2012;28: 2520–2522. doi:[10.1093/bioinformatics/bts480](https://doi.org/10.1093/bioinformatics/bts480)

21. Klimanskaya I, Hipp J, Rezai KA, West M, Atala A, Lanza R. Derivation and comparative assessment of retinal pigment epithelium from human embryonic stem cells using transcriptomics. Cloning and Stem Cells. 2004;6: 217–245. doi:[10.1089/clo.2004.6.217](https://doi.org/10.1089/clo.2004.6.217)

22. Smith EN, D’Antonio-Chronowska A, Greenwald WW, Borja V, Aguiar LR, Pogue R, et al. Human iPSC-Derived Retinal Pigment Epithelium: A Model System for Prioritizing and Functionally Characterizing Causal Variants at AMD Risk Loci. Stem Cell Reports. 2019;12: 1342–1353. doi:[10.1016/j.stemcr.2019.04.012](https://doi.org/10.1016/j.stemcr.2019.04.012)

23. Zerbino DR, Achuthan P, Akanni W, Amode MR, Barrell D, Bhai J, et al. Ensembl 2018. Nucleic Acids Research. 2018;46: D754–D761. doi:[10.1093/nar/gkx1098](https://doi.org/10.1093/nar/gkx1098)

24. O’Leary NA, Wright MW, Brister JR, Ciufo S, Haddad D, McVeigh R, et al. Reference sequence (RefSeq) database at NCBI: Current status, taxonomic expansion, and functional annotation. Nucleic Acids Research. 2016;44: D733–745. doi:[10.1093/nar/gkv1189](https://doi.org/10.1093/nar/gkv1189)

25. Landry J-R, Mager DL, Wilhelm BT. Complex controls: The role of alternative promoters in mammalian genomes. Trends in Genetics. 2003;19: 640–648. doi:[10.1016/j.tig.2003.09.014](https://doi.org/10.1016/j.tig.2003.09.014)

26. Tian B, Manley JL. Alternative polyadenylation of mRNA precursors. Nature Reviews Molecular Cell Biology. 2017;18: 18–30. doi:[10.1038/nrm.2016.116](https://doi.org/10.1038/nrm.2016.116)

27. Wang Y, Liu J, HUANG B, XU Y-M, LI J, HUANG L-F, et al. Mechanism of alternative splicing and its regulation. Biomedical Reports. 2015;3: 152–158. doi:[10.3892/br.2014.407](https://doi.org/10.3892/br.2014.407)

28. Pollard KS, Hubisz MJ, Rosenbloom KR, Siepel A. Detection of nonneutral substitution rates on mammalian phylogenies. Genome Research. 2010;20: 110–121. doi:[10.1101/gr.097857.109](https://doi.org/10.1101/gr.097857.109)

29. Noguchi S, Arakawa T, Fukuda S, Furuno M, Hasegawa A, Hori F, et al. FANTOM5 CAGE profiles of human and mouse samples. Scientific Data. 2017;4: 170112. doi:[10.1038/sdata.2017.112](https://doi.org/10.1038/sdata.2017.112)

30. Herrmann CJ, Schmidt R, Kanitz A, Artimo P, Gruber AJ, Zavolan M. PolyASite 2.0: A consolidated atlas of polyadenylation sites from 3′ end sequencing. Nucleic Acids Research. 2020;48: D174–D179. doi:[10.1093/nar/gkz918](https://doi.org/10.1093/nar/gkz918)

31. Takahashi H, Kato S, Murata M, Carninci P. CAGE- Cap Analysis Gene Expression: A protocol for the detection of promoter and transcriptional networks. Methods in molecular biology (Clifton, NJ). 2012;786: 181–200. doi:[10.1007/978-1-61779-292-2\_11](https://doi.org/10.1007/978-1-61779-292-2_11)

32. Beck AH, Weng Z, Witten DM, Zhu S, Foley JW, Lacroute P, et al. 3′-End Sequencing for Expression Quantification (3SEQ) from Archival Tumor Samples. PLOS ONE. 2010;5: e8768. doi:[10.1371/journal.pone.0008768](https://doi.org/10.1371/journal.pone.0008768)

33. Bharti K, Liu W, Csermely T, Bertuzzi S, Arnheiter H. Alternative promoter use in eye development: Complex role and regulation of the transcription factor MITF. Development (Cambridge, England). 2008;135: 1169–1178. doi:[10.1242/dev.014142](https://doi.org/10.1242/dev.014142)

34. Mellough CB, Bauer R, Collin J, Dorgau B, Zerti D, Dolan DWP, et al. An integrated transcriptional analysis of the developing human retina. Development (Cambridge, England). 2019;146. doi:[10.1242/dev.169474](https://doi.org/10.1242/dev.169474)

35. Christ A, Christa A, Klippert J, Eule JC, Bachmann S, Wallace VA, et al. LRP2 Acts as SHH Clearance Receptor to Protect the Retinal Margin from Mitogenic Stimuli. Developmental Cell. 2015;35: 36–48. doi:[10.1016/j.devcel.2015.09.001](https://doi.org/10.1016/j.devcel.2015.09.001)

36. Braun TA, Mullins RF, Wagner AH, Andorf JL, Johnston RM, Bakall BB, et al. Non-exomic and synonymous variants in ABCA4 are an important cause of Stargardt disease. Human Molecular Genetics. 2013;22: 5136–5145. doi:[10.1093/hmg/ddt367](https://doi.org/10.1093/hmg/ddt367)

37. Bauwens M, Garanto A, Sangermano R, Naessens S, Weisschuh N, De Zaeytijd J, et al. ABCA4-associated disease as a model for missing heritability in autosomal recessive disorders: Novel noncoding splice, cis-regulatory, structural, and recurrent hypomorphic variants. Genetics in Medicine. 2019;21: 1761–1771. doi:[10.1038/s41436-018-0420-y](https://doi.org/10.1038/s41436-018-0420-y)

38. Zernant J, Xie Y(, Ayuso C, Riveiro-Alvarez R, Lopez-Martinez M-A, Simonelli F, et al. Analysis of the ABCA4 genomic locus in Stargardt disease. Human Molecular Genetics. 2014;23: 6797–6806. doi:[10.1093/hmg/ddu396](https://doi.org/10.1093/hmg/ddu396)

39. Sangermano R, Garanto A, Khan M, Runhart EH, Bauwens M, Bax NM, et al. Deep-intronic ABCA4 variants explain missing heritability in Stargardt disease and allow correction of splice defects by antisense oligonucleotides. Genetics in Medicine. 2019;21: 1751–1760. doi:[10.1038/s41436-018-0414-9](https://doi.org/10.1038/s41436-018-0414-9)

40. Jamshidi F, Place EM, Mehrotra S, Navarro-Gomez D, Maher M, Branham KE, et al. Contribution of non-coding mutations to RPGRIP1-mediated inherited retinal degeneration. Genetics in medicine : official journal of the American College of Medical Genetics. 2019;21: 694–704. doi:[10.1038/s41436-018-0104-7](https://doi.org/10.1038/s41436-018-0104-7)

41. Mayer AK, Rohrschneider K, Strom TM, Glöckle N, Kohl S, Wissinger B, et al. Homozygosity mapping and whole-genome sequencing reveals a deep intronic PROM1 mutation causing cone–rod dystrophy by pseudoexon activation. European Journal of Human Genetics. 2016;24: 459–462. doi:[10.1038/ejhg.2015.144](https://doi.org/10.1038/ejhg.2015.144)

42. Geoffroy V, Stoetzel C, Scheidecker S, Schaefer E, Perrault I, Bär S, et al. Whole-genome sequencing in patients with ciliopathies uncovers a novel recurrent tandem duplication in IFT140. Human Mutation. 2018;39: 983–992. doi:[10.1002/humu.23539](https://doi.org/10.1002/humu.23539)

43. Lenis TL, Hu J, Ng SY, Jiang Z, Sarfare S, Lloyd MB, et al. Expression of ABCA4 in the retinal pigment epithelium and its implications for Stargardt macular degeneration. Proceedings of the National Academy of Sciences. 2018;115: E11120–E11127. doi:[10.1073/pnas.1802519115](https://doi.org/10.1073/pnas.1802519115)

44. Reyes A, Huber W. Alternative start and termination sites of transcription drive most transcript isoform differences across human tissues. Nucleic Acids Research. 2018;46: 582–592. doi:[10.1093/nar/gkx1165](https://doi.org/10.1093/nar/gkx1165)

45. Yan W, Peng Y-R, Zyl T van, Regev A, Shekhar K, Juric D, et al. Cell Atlas of The Human Fovea and Peripheral Retina. Scientific Reports. 2020;10: 9802. doi:[10.1038/s41598-020-66092-9](https://doi.org/10.1038/s41598-020-66092-9)

46. Bryan JM, Fufa TD, Bharti K, Brooks BP, Hufnagel RB, McGaughey DM. Identifying core biological processes distinguishing human eye tissues with precise systems-level gene expression analyses and weighted correlation networks. Human Molecular Genetics. 2018;27: 3325–3339. doi:[10.1093/hmg/ddy239](https://doi.org/10.1093/hmg/ddy239)

47. May-Simera HL, Wan Q, Jha BS, Hartford J, Khristov V, Dejene R, et al. Primary Cilium-Mediated Retinal Pigment Epithelium Maturation Is Disrupted in Ciliopathy Patient Cells. Cell reports. 2018;22: 189–205. doi:[10.1016/j.celrep.2017.12.038](https://doi.org/10.1016/j.celrep.2017.12.038)

48. Kelley RA, Chen HY, Swaroop A, Li T. Accelerated Development of Rod Photoreceptors in Retinal Organoids Derived from Human Pluripotent Stem Cells by Supplementation with 9-cis Retinal. STAR protocols. 2020;1. doi:[10.1016/j.xpro.2020.100033](https://doi.org/10.1016/j.xpro.2020.100033)

49. Ohlemacher SK, Iglesias CL, Sridhar A, Gamm DM, Meyer JS. Generation of highly enriched populations of optic vesicle-like retinal cells from human pluripotent stem cells. Current Protocols in Stem Cell Biology. 2015;32: 1H.8.1–1H.8.20. doi:[10.1002/9780470151808.sc01h08s32](https://doi.org/10.1002/9780470151808.sc01h08s32)

50. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: Ultrafast universal RNA-seq aligner. Bioinformatics (Oxford, England). 2013;29: 15–21. doi:[10.1093/bioinformatics/bts635](https://doi.org/10.1093/bioinformatics/bts635)

51. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics (Oxford, England). 2009;25: 2078–2079. doi:[10.1093/bioinformatics/btp352](https://doi.org/10.1093/bioinformatics/btp352)

52. Pertea G, Pertea M. GFF Utilities: GffRead and GffCompare. F1000Research. 2020;9: 304. doi:[10.12688/f1000research.23297.1](https://doi.org/10.12688/f1000research.23297.1)

53. Patro R, Duggal G, Love MI, Irizarry RA, Kingsford C. Salmon provides fast and bias-aware quantification of transcript expression. Nature methods. 2017;14: 417–419. doi:[10.1038/nmeth.4197](https://doi.org/10.1038/nmeth.4197)

54. Neph S, Kuehn MS, Reynolds AP, Haugen E, Thurman RE, Johnson AK, et al. BEDOPS: High-performance genomic feature operations. Bioinformatics. 2012;28: 1919–1920. doi:[10.1093/bioinformatics/bts277](https://doi.org/10.1093/bioinformatics/bts277)

55. Quinlan AR, Hall IM. BEDTools: A flexible suite of utilities for comparing genomic features. Bioinformatics (Oxford, England). 2010;26: 841–842. doi:[10.1093/bioinformatics/btq033](https://doi.org/10.1093/bioinformatics/btq033)

56. Lex A, Gehlenborg N, Strobelt H, Vuillemot R, Pfister H. UpSet: Visualization of Intersecting Sets. IEEE Transactions on Visualization and Computer Graphics. 2014;20: 1983–1992. doi:[10.1109/TVCG.2014.2346248](https://doi.org/10.1109/TVCG.2014.2346248)

57. Allen M, Poggiali D, Whitaker K, Marshall TR, Kievit RA. Raincloud plots: A multi-platform tool for robust data visualization. Wellcome Open Research. 2019;4: 63. doi:[10.12688/wellcomeopenres.15191.1](https://doi.org/10.12688/wellcomeopenres.15191.1)

58. Robinson MD, McCarthy DJ, Smyth GK. edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 2010;26: 139–140. doi:[10.1093/bioinformatics/btp616](https://doi.org/10.1093/bioinformatics/btp616)

59. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. Limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Research. 2015;43: e47–e47. doi:[10.1093/nar/gkv007](https://doi.org/10.1093/nar/gkv007)

60. Yu G, Wang L-G, Han Y, He Q-Y. clusterProfiler: An R Package for Comparing Biological Themes Among Gene Clusters. OMICS : a Journal of Integrative Biology. 2012;16: 284–287. doi:[10.1089/omi.2011.0118](https://doi.org/10.1089/omi.2011.0118)

61. Gu Z, Eils R, Schlesner M. Complex heatmaps reveal patterns and correlations in multidimensional genomic data. Bioinformatics. 2016;32: 2847–2849. doi:[10.1093/bioinformatics/btw313](https://doi.org/10.1093/bioinformatics/btw313)

62. Zhao H, Sun Z, Wang J, Huang H, Kocher J-P, Wang L. CrossMap: A versatile tool for coordinate conversion between genome assemblies. Bioinformatics (Oxford, England). 2014;30: 1006–1007. doi:[10.1093/bioinformatics/btt730](https://doi.org/10.1093/bioinformatics/btt730)

63. McLaren W, Gil L, Hunt SE, Riat HS, Ritchie GRS, Thormann A, et al. The Ensembl Variant Effect Predictor. Genome Biology. 2016;17: 122. doi:[10.1186/s13059-016-0974-4](https://doi.org/10.1186/s13059-016-0974-4)

64. R Core Team. R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing; 2019. Available: <https://www.R-project.org/>